Prevention of *Escherichia coli* Respiratory Infection in Broiler Chickens with Bacteriophage (SPR02)[1]

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ABSTRACT Bacteriophages are viruses that can infect and kill bacteria. Three studies were conducted to determine the efficacy of bacteriophage to prevent an *Escherichia coli* respiratory infection in broiler chickens. In the first study 3-d-old-birds were challenged with an air sac inoculation of 10^3 cfu of *E. coli* per mL mixed with either 10^3 or 10^6 pfu of bacteriophage, or 10^4 cfu *E. coli* mixed with 10^4 or 10^8 pfu of bacteriophage. In the second study, drinking water of birds to 1 wk of age was treated with 10^3 or 10^4 pfu of bacteriophage per mL and birds were air sac challenged with 10^3 cfu of *E. coli*, or water was treated with 10^4 or 10^6 pfu of bacteriophage per milliliter and birds were challenged with 10^4 cfu of *E. coli*. In the third study, birds were air sac challenged at 1 wk of age with 10^4 cfu of *E. coli* and given 10^5 or 10^6 pfu of bacteriophage per mL of water from 1 d of age to 2 wk of age. In Studies 1 and 2, there were two replicate pens per treatment with 10 birds per pen, and in Study 3, there were four replicate pens per treatment with 10 birds per pen. The studies were all concluded when the birds were 3 wk of age. In Study 1, BW was decreased at 1 and 2 wk of age in the birds that were challenged with 10^3 or 10^4 cfu of *E. coli* and was decreased at 2 wk of age in the birds challenged with 10^4 cfu of *E. coli* mixed with 10^4 pfu of the bacteriophage. Mortality was decreased from 80% in the birds challenged with 10^3 cfu of *E. coli* to 25 and 5% when mixed with 10^3 or 10^6 pfu of the bacteriophage, respectively. Mortality was decreased from 85% in birds challenged with 10^4 cfu of *E. coli* to 35% when mixed with 10^4 pfu of the bacteriophage, and no mortality occurred when mixed with 10^8 pfu of bacteriophage. There was essentially no protection observed in Studies 2 and 3 when the birds were challenged with 10^3 or 10^4 cfu of *E. coli* with bacteriophage present in their drinking water at any level. These data suggest that bacteriophage can protect birds from a respiratory challenge with *E. coli*, but that adding the bacteriophage to the drinking water offered no protection to the birds. The complete protection of the birds observed in Study 1 suggests that bacteriophage may possibly be developed as an alternative to antibiotic use in poultry.

(*Key words*: bacteriophage, *Escherichia coli*, chicken)

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INTRODUCTION

The emergence of pathogenic bacteria with resistance to multiple antibiotics has reduced the effectiveness of antibiotic treatment of infections. As cases of infection caused by antibiotic resistant bacteria increase, there is growing concern over the use of antibiotics in both human and animal medicine. Of particular concern, to some, is the possible contribution to the emergence of antibiotic resistant bacteria that may result from the use of therapeutic and subtherapeutic levels of antibiotics in animal production (Levy, 1998). The importance of the use of antibiotics in animal production to the emergence of antibiotic resistant bacteria that cause human infections is equivocal. However, these concerns may result in regulations and laws severely restricting or banning the use of antibiotics in animal production. Colibacillosis is considered the most common cause of death and condemnations in the poultry industry (Piercy and West, 1976; DeRosa et al., 1992), necessitating the use of antibiotics targeted at *Escherichia coli*. Therefore, there is a real need to find alternatives to antibiotics for both the prevention and treatment of *E. coli* infections in poultry.

Bacteriophages are viruses that can kill bacteria and it may be possible to use these viruses to both prevent and treat bacterial diseases. Bacteriophages were independently described by Twort (1915) and d'Herelle (1917). Recently there has been renewed interest in using bacteriophage to treat or prevent bacterial infections, but little work is reported in poultry. H. W. Smith published several papers demonstrating efficacy of bacteriophage to control

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E. coli-induced diarrhea in calves, piglets, and lambs (Smith and Huggins, 1983; Smith et al., 1987) and to treat *E. coli* infections in mice (Smith and Huggins, 1982). Barrow et al. (1998) demonstrated that bacteriophage could effectively prevent and treat *E. coli*-induced septicemia and meningitis in chickens with a bacteriophage, and Soothill (1992) demonstrated protection in mice from infection with *Acinetobacter baumanii* and *Pseudomonas aeruginosa*. Extensive Russian literature exists on the use of bacteriophage in treating human diseases, work conducted primarily at the Eliava Institute in Georgia, some of which is reviewed in an article by Alisky et al. (1998). There are also several papers published from the Institute of Immunology and Experimental Therapy, at the Polish Academy of Sciences on the use of bacteriophage in human medicine (Ślopek et al., 1981, 1984, 1985, 1987; Weber-Dąbrowska et al., 1987), and there is an excellent review on the use of bacteriophage as an alternative to antibiotics published on the internet (Kutter, 1997).

There is a real need for the poultry industry to find alternatives to antibiotics to prevent or treat significant poultry diseases, such as colibacillosis, as well as reduce the levels of food borne pathogens on poultry products. The objectives of this work were to isolate bacteriophage to an *E. coli* strain pathogenic in poultry and to investigate the efficacy of these bacteriophages to prevent *E. coli* infections in broiler chickens.

MATERIALS AND METHODS

Bacteriophage Isolation

Water samples were collected from two municipal sewer treatment plants and a poultry processing plant. The water samples were passed through coarse and fine Buchner filters, and sterilized by filtering through 0.2-μm membrane filters after wetting the filters with 10% fetal calf serum. Enrichment for bacteriophage was conducted by adding a loop of *E. coli* to 5 mL of double strength tryptose phosphate broth[3] containing 10 mM $MgSO_4$, which was incubated at 37 C for 2.5 h. Five milliliters of the environmental water samples was added to the tubes, and samples were incubated for 24 h at 37 C. After incubation, the tubes were centrifuged at 2,000 $\times$ *g* for 15 min. Bacteriophages were isolated from these enrichment tubes by serially diluting the supernatant and preparing soft agar overlay plates. The tryptose phosphate soft agar was prepared to a final concentration of 0.65% agar, 2.95% tryptose phosphate broth, and 5 mM $MgSO_4$ in 2.5 mL. The procedure was to pre-incubate tryptose phosphate agar plates at 45 C to dry the agar surface. Next 100 μL of a 2.5-h *E. coli* stock culture (approximately 10^8 cfu/mL) was added, as was 1 mL of the enrichment supernatant to 1.5 mL tryptose phosphate soft agar tubes. The tubes were mixed and then poured over the tryptose phosphate agar plates. The soft agar plates were incubated at 37 C overnight. Clear zones representing bacteriophage plaques were apparent. These plaques were transferred by stabbing the plaques with an inoculation needle, washing the needle in sterile PBS, and plating on soft agar plates as described above. Bacteriophages were purified by transferring the plaques a minimum of three times. With these procedures we isolated and purified 18 bacteriophages. Based on the relatively large size and degree of clearing of the plaques, one bacteriophage (SPR02) was selected to determine its efficacy to prevent *E. coli* respiratory infections in broiler chickens.

Bacteriophage Amplification

Bacteriophage (SPR02) was amplified by preparing soft agar plates as described above and inoculating with 1 mL of approximately 10^3 bacteriophage. The plates were incubated for 48 h and then flooded with 10 mL of PBS. The flooded plates were maintained for 4 h with occasional gentle mixing. The liquid was removed from the plates and centrifuged at 2,500 $\times$ *g* for 15 min, and the supernatant was sterilized using 0.2-μm syringe filters. The bacteriophages were enumerated by making serial dilutions and preparing soft agar overlay plates. This procedure consistently produced bacteriophage preparations containing 10^9 bacteriophage/mL.

Experimental Design

Three studies were conducted to determine the efficacy of SPR02 to prevent *E. coli* respiratory infection in broiler chickens. Study 1 consisted of 13 treatments with 2 replicate pens of 10 birds per pen. The birds were maintained in electrically heated batteries with feed and water available ad libitum to 3 wk of age when the experiment was concluded. Two treatments consisted of birds challenged with either 2.16×10^3 or 1.12×10^4 cfu *E. coli* injected (0.1 mL) into the thoracic air sac when the birds were 3 d of age. Four treatments consisted of mixing the 10^3 *E. coli* challenge culture with either 10^3 or 10^6 bacteriophage, and mixing the 10^4 *E. coli* challenge culture with 10^4 or 10^8 bacteriophage 24 h prior to challenge. These preparations were kept refrigerated during this period. After 24 h, there was no difference in viable *E. coli* plate counts of any of the combinations of the bacteriophage-*E. coli* mixtures compared to the untreated *E. coli* challenge cultures. Four treatments consisted of challenging the birds with 10^3, 10^4, 10^6, or 10^8 bacteriophage. There were three control treatments consisting of unchallenged birds, birds challenged with the culture medium, or birds challenged with PBS.

The birds and feed were weighed each week. Any birds that died were weighed; severity of airsaculitis was scored (Huff et al., 1998), and the liver and air sac were cultured with sterile swabs and plated on McConkey's agar,[4] with isolates compared to the inoculum culture based on colony morphology and biochemical characteristics. The liver, heart, spleen, and bursa of Fabricius were excised and weighed. When the birds were 3 wk of age they were

[3]Sigma Chemical Co., St. Louis, MO.
[4]Remel, Lenexa, KS.

killed by cervical dislocation and necropsied as described for the mortalities.

In Study 2, there were 12 treatments with 2 replicate pens of 10 birds per pen. Two treatments consisted of birds challenged at 1 wk of age with 2.16×10^3 or 1.12×10^4 cfu *E. coli* by injecting 0.1 mL into the thoracic air sac. Four treatments consisted of having 10^3 or 10^6 bacteriophage per milliliter in the drinking water from 1 d of age to 1 wk of age when the birds were challenged with 2.16×10^3 cfu of *E. coli*, and 10^4 or 10^6 bacteriophage per milliliter in the drinking water challenged at 1 wk of age with 1.12×10^4 cfu of *E. coli*. Three treatments consisted of birds unchallenged with 10^3, 10^4, or 10^6 bacteriophage per milliliter in their drinking water to 1 wk of age. The controls and all other experimental conditions and procedures were as described for Study 1.

In Study 3, there were seven treatments with four replicate pens of 10 birds per pen. One treatment consisted of birds challenged with a 0.1 mL injection into the thoracic airsac of 2.29×10^4 cfu of *E. coli* when the birds were 1 wk of age. Two treatments consisted of 10^5 or 10^6 bacteriophage per milliliter in the drinking water from 1 d of age to 2 wk of age and the birds challenged at 1 wk of age with 2.29×10^4 cfu of *E. coli*. Two treatments consisted of birds unchallenged with 10^5 or 10^6 bacteriophage per milliliter in their drinking water. Two control treatments consisted of unchallenged birds on normal water and birds challenged with PBS on normal water. All other experimental conditions and procedures were as described for Study 1.

Statistical Analysis

These data were analyzed by ANOVA (Snedecor and Cochran, 1967), using the General Linear Models procedures of SAS® software (SAS Institute, 1988). All data presented as percentages were arc sine $\sqrt{\%}$ transformed prior to statistical analysis. Significant differences between treatments were separated using Duncan's multiple-range test (Duncan, 1955). All statements of significance are based on the probability level of 0.05.

RESULTS

The effects on BW of mixing bacteriophage (SPR02) with the *E. coli* challenge culture prior to challenging the birds (Study 1) are presented in Table 1. Body weights were significantly decreased from the control treatment at 1 and 2 wk in the birds challenged with 10^3 or 10^4 cfu of *E. coli* and at 2 wk in the birds challenged with 10^4 of *E. coli* mixed with 10^4 pfu of the bacteriophage. The effects of these treatments on mortality are shown in Table 2. There was significant mortality (80%) in the birds challenged with 10^3 cfu of *E. coli*. Mixing the 10^3 culture with 10^3 or 10^6 pfu of bacteriophage resulted in a significant reduction in mortality to 25 and 5%, respectively. There was 85% mortality when birds were challenged with 10^4 cfu of *E. coli*, and a significant reduction in mortality when mixed with 10^4 pfu of bacteriophage (35%) and no mortality when the culture was mixed with 10^8 pfu of the bacteriophage. Necropsy of the surviving birds did not show any consistent treatment effects on the relative weights of the liver, heart, spleen, and bursa of Fabricius, the lesion scores, and the lack of positive isolates from the livers and air sacs of these birds suggesting that the surviving birds had cleared this *E. coli* challenge (data not shown). The birds that died during this study in the *E. coli*-challenged groups consistently had enlarged livers, hearts, spleens, and reduced bursa of Fabricius relative weights, the air sac lesion scores were high, and the challenge organism could be isolated from the liver and air sac of these birds (data not shown).

The effect on BW of treating the drinking water with bacteriophage (SPR02) 1 week prior to challenge with *E. coli* (Study 2) are shown in Table 3. At 2 wk of age there was a significant decrease in BW from the control treatment when birds were challenged with 10^3 cfu of *E. coli* on untreated water, and in all the birds challenged with 10^4 cfu of *E. coli* regardless of water treatment. At 3 wk of age

TABLE 1. The effect of *Escherichia coli* challenge with and without mixing the challenge culture with bacteriophage (SPR02) on body weights

Treatment	1 wk (g)	2 wk (g)	3 wk (g)
Control	135 ± 7^{abc}	384 ± 10^{a}	757 ± 16^{ab}
Broth	143 ± 4^{ab}	380 ± 9^{a}	723 ± 15^{ab}
PBS	150 ± 3^{a}	356 ± 12^{a}	758 ± 15^{ab}
Phage 10^3	133 ± 6^{abc}	382 ± 8^{a}	733 ± 11^{ab}
Phage 10^4	120 ± 6^{c}	345 ± 8^{a}	712 ± 20^{ab}
Phage 10^6	130 ± 9^{bc}	388 ± 10^{a}	786 ± 11^{a}
Phage 10^8	124 ± 7^{bc}	353 ± 13^{a}	727 ± 24^{ab}
E. coli 10^3	97 ± 7^{d}	195 ± 36^{d}	683 ± 133^{b}
E. coli 10^3 + phage 10^3	136 ± 7^{abc}	344 ± 29^{a}	743 ± 37^{ab}
E. coli 10^3 + phage 10^6	123 ± 7^{c}	360 ± 12^{a}	726 ± 15^{ab}
E. coli 10^4	84 ± 6^{d}	240 ± 57^{cd}	685 ± 158^{b}
E. coli 10^4 + phage 10^4	120 ± 7^{c}	282 ± 30^{bc}	735 ± 52^{ab}
E. coli 10^4 + phage 10^8	121 ± 4^{c}	332 ± 9^{ab}	684 ± 12^{b}

[a-d]Values represent the mean ± SEM of two replicate pens of 10 birds per pen. Values within a column with different superscripts differ significantly ($P \leq 0.05$).

TABLE 2. The effect of *Escherichia coli* challenge with and without mixing the challenge culture with bacteriophage (SPR02) on mortality

Treatment	Mortality (n died / total; %)
Control	1/20^{c} (5)
Broth	0/20^{c} (0)
PBS	0/20^{c} (0)
Phage 10^3	1/20^{c} (5)
Phage 10^4	1/20^{c} (5)
Phage 10^6	2/20^{c} (10)
Phage 10^8	1/20^{c} (5)
E. coli 10^3	16/20^{a} (80)
E. coli 10^3 + phage 10^3	5/20^{bc} (25)
E. coli 10^3 + phage 10^6	1/20^{c} (5)
E. coli 10^4	17/20^{a} (85)
E. coli 10^4 + phage 10^4	7/20^{b} (35)
E. coli 10^4 + phage 10^8	0/20^{c} (0)

[a-c]Values represent the mean ± SEM of two replicate pens of 10 birds per pen. Values within a column with different superscripts differ significantly ($P \leq 0.05$).

TABLE 3. The effect of *Escherichia coli* challenge with and without bacteriophage (SPR02) present in drinking water prior to challenge on body weights

Treatment	1 wk (g)	2 wk (g)	3 wk (g)
Control	135 ± 7^{abcd}	384 ± 10^{a}	757 ± 16^{a}
Broth	143 ± 4^{abcd}	380 ± 9^{a}	723 ± 15^{ab}
PBS	150 ± 3^{ab}	356 ± 12^{ab}	758 ± 15^{a}
Phage 10^3	148 ± 5^{abc}	375 ± 9^{a}	642 ± 10^{b}
Phage 10^4	129 ± 5^{d}	353 ± 16^{ab}	720 ± 13^{ab}
Phage 10^6	146 ± 6^{abcd}	403 ± 9^{a}	735 ± 14^{ab}
E. coli 10^3	133 ± 6^{bcd}	286 ± 22^{c}	699 ± 45^{ab}
E. coli 10^3 + phage 10^3	142 ± 4^{abcd}	343 ± 29^{ab}	749 ± 24^{a}
E. coli 10^3 + phage 10^6	131 ± 9^{cd}	359 ± 26^{ab}	736 ± 41^{ab}
E. coli 10^4	135 ± 7^{abcd}	258 ± 27^{c}	674 ± 65^{ab}
E. coli 10^4 + phage 10^4	152 ± 5^{a}	310 ± 26^{bc}	639 ± 66^{b}
E. coli 10^4 + phage 10^6	144 ± 3^{abcd}	284 ± 26^{c}	696 ± 38^{ab}

$^{a-d}$Values represent the mean ± SEM of two replicate pens of 10 birds per pen. Values within a column with different superscripts differ significantly ($P \leq 0.05$).

only the birds receiving the 10^3 pfu water treatment and the birds challenged with 10^4 cfu of *E. coli* with 10^4 pfu of bacteriophage in their drinking water had BW significantly less than the control treatment. The effect of these treatments on mortality is shown in Table 4. Mortality was significantly increased from the control treatment only in the birds that received 10^4 cfu of *E. coli* with untreated water. As in the previous study, no lesions consistent with *E. coli* infection were detected at necropsy, whereas the birds that died during this study had lesions consistent with severe colisepticemia (data not shown).

The effect of treating the drinking water birds with bacteriophage (SPR02) on BW 1 wk prior to challenge with 10^4 cfu of *E. coli* and continuing 1 wk after challenge are shown in Table 5. At 2 wk, all birds challenged with 10^4 cfu of *E. coli*, regardless of water treatment, had significantly reduced BW compared to the control treatment, and at 3 wk only the birds challenged with 10^4 cfu of *E. coli* with 10^6 pfu of the bacteriophage had BW significantly different from the control birds. The effects of these treatments on mortality are shown in Table 6. All birds challenged with

TABLE 4. The effect of *Escherichia coli* challenge with and without bacteriophage (SPR02) present in drinking water prior to challenge on mortality

Treatment	Mortality (n died / total; %)
Control	1/20^{b} (5)
Broth	0/20^{b} (0)
PBS	0/20^{b} (0)
Phage 10^3	0/20^{b} (0)
Phage 10^4	1/20^{b} (5)
Phage 10^6	1/20^{b} (5)
E. coli 10^3	6/20^{ab} (30)
E. coli 10^3 + phage 10^3	5/20^{ab} (25)
E. coli 10^3 + phage 10^6	6/20^{ab} (30)
E. coli 10^4	11/20^{a} (55)
E. coli 10^4 + phage 10^4	7/20^{ab} (35)
E. coli 10^4 + phage 10^6	8/20^{ab} (40)

^{a,b}Values represent the mean ± SEM of two replicate pens of 10 birds per pen. Values within a column with different superscripts differ significantly ($P \leq 0.05$).

TABLE 5. The effect of *Escherichia coli* challenge with and without bacteriophage (SPR02) present in the drinking 1 wk prior to challenge and continuing 1 wk after challenge on body weights

Treatment	1 wk (g)	2 wk (g)	3 wk (g)
Control	154 ± 3^{a}	405 ± 6^{a}	734 ± 9^{ab}
PBS	148 ± 3^{a}	399 ± 7^{ab}	743 ± 16^{ab}
Phage 10^5	154 ± 3^{a}	396 ± 5^{ab}	705 ± 8^{bc}
Phage 10^6	153 ± 3^{a}	402 ± 6^{ab}	709 ± 8^{bc}
E. coli 10^4	148 ± 3^{a}	352 ± 23^{c}	781 ± 52^{a}
E. coli 10^4 + phage 10^5	156 ± 2^{a}	373 ± 14^{bc}	695 ± 30^{bc}
E. coli 10^4 + phage 10^6	155 ± 3^{a}	271 ± 20^{d}	653 ± 15^{c}

$^{a-c}$Values represent the mean ± SEM of four replicate pens of 10 birds per pen. Values within a column with different superscripts differ significantly ($P \leq 0.05$).

10^4 cfu of *E. coli* regardless of water treatment had significantly increased mortality from the control treatment. As in the two previous studies, the birds that died had typical *E. coli* respiratory and septicemic lesions, the livers and air sacs were culture positive, whereas the birds that were necropsied were generally culture negative and had little or no lesions consistent with *E. coli* infection.

DISCUSSION

These studies demonstrate both the promise and the frustration of using bacteriophage to either prevent or treat infectious diseases. We were able to completely protect birds from an *E. coli* respiratory challenge when the challenge culture was mixed with bacteriophage 24 h before challenging the birds, reducing mortality from around 80 to 0%. Although this experimental design was artificial, it suggests that if appropriate bacteriophage are present, at the right time, and at the right titer, they may prevent infection by the organism targeted by the bacteriophage, in this case *E. coli*. However, we saw little or no efficacy to prevent infection when high titers of bacteriophage were present in the drinking water and the birds challenged with a large inoculum of *E. coli* injected directly into the thoracic air sac.

Although there are few reports on the use of bacteriophage as an alternative to antibiotics, and almost no literature in poultry, there has been enough success of bacterio-

TABLE 6. The effect of *Escherichia coli* challenge with and without bacteriophage (SPR02) present in drinking water 1 wk prior to challenge and continuing 1 wk after challenge on mortality

Treatment	Mortality (n died / total; %)
Control	0/40^{c} (0)
PBS	1/40^{c} (2.5)
Phage 10^5	1/40^{c} (2.5)
Phage 10^6	0/40^{c} (0)
E. coli 10^4	30/40^{ab} (75)
E. col 10^4 + phage 10^5	24/40^{b} (60)
E. coli 10^4 + phage 10^6	36/40^{a} (90)

$^{a-c}$Values represent the mean ± SEM of four replicate pens of 10 birds per pen. Values within a column with different superscripts differ significantly ($P \leq 0.05$).

phage in other animal model systems to support the hypothesis that bacteriophage may have efficacy to prevent diseases. Barrow et al. (1998) reported significant protection in chickens from an intramuscular injection of *E. coli* when simultaneously injected at a different site with a bacteriophage, and some protection when *E. coli* was injected intracranially and the bacteriophage was simultaneously injected intramuscularly. Smith and Huggins (1983) demonstrated that calves orally challenged with *E. coli* and given oral doses of bacteriophage 1 or 8 h after challenge were protected by the bacteriophage. In addition, Smith et al. (1987) demonstrated efficacy of bacteriophage to prevent calves from contracting *E. coli* diarrhea when challenged orally and given bacteriophage in their milk feeds up to 8 h after the challenge, and could protect calves orally challenged with *E. coli* and maintained in rooms in which the litter was treated with bacteriophage.

The failure of protecting the birds by treating water with bacteriophage from a large inoculation of *E. coli* directly into the thoracic air sac was expected. It was not anticipated that the birds would respire enough bacteriophage from their drinking water to protect them from such a severe challenge. We wanted to try this approach because, if successful, it would be a practical method to administer bacteriophage in the poultry industry. In addition, the bacteriophage selected for these studies may not be the most efficacious bacteriophage against this particular *E. coli* and there is a real need to develop in vitro tests to screen large numbers of bacteriophage isolates for prophylactic or therapeutic efficacy against bacterial diseases.

In summary, these studies suggest that bacteriophage may have efficacy to prevent poultry diseases and provide an alternative to antibiotics. However, a great deal of work is needed to isolate bacteriophage effective against a specific target bacteria, establish criteria to select the most efficacious bacteriophage, and to determine how, when, and how much bacteriophage would be needed to provide adequate protection to poultry from a specific bacterial target. Most if not all of these considerations must be established for each bacteria believed to pose a risk to poultry health. In addition, the specificity of bacteriophages to selected serotypes within a species would seem to also be a difficult hurdle to overcome. The advantages of using bacteriophages to prevent diseases are that they are natural, plentiful, relatively inexpensive to amplify, and their effectiveness may be enhanced by genetic manipulation.

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